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ZINC OXIDE NANOPARTICLES FOR IMMUNITY ENHANCEMENT AND BACTERIAL DISEASE CONTROL IN FISH FARMING

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Abstract

This study examines the efficacy of green-synthesized zinc oxide nanoparticles (ZnO NPs) as an immunostimulant and antibacterial agent in aquaculture. Citrus limetta leaf extract was used for the synthesis of ZnO NPs, which were then studied using UV-Vis spectroscopy, FTIR, XRD, SEM, and zeta potential analysis to confirm their stability, crystalline structure, nanoscale size, and functional surface characteristics. Oreochromis niloticus experienced diets supplemented with ZnO NPs at 0, 0.5, 1.0, and 2.0 mg/kg for 60 days as part of an in vivo investigation. Results revealed that the 1.0 mg/kg dose (Group III) significantly improved innate immune measurements, including lysozyme activity, respiratory burst, and serum protein levels, as well as growth performance and hematological indices. Following a challenge with Aeromonas hydrophila, this group exhibited the highest survival rate (90%), lowest bacterial load in tissues, and minimal histopathological damage, confirming robust disease resistance. Higher doses showed reduced efficacy, indicating an optimal threshold. These findings establish ZnO NPs as a sustainable alternative to antibiotics, effectively enhancing immunity and controlling bacterial diseases in fish farming.

INTRODUCTION

The global aquaculture industry stands as a critical pillar of food security, projected to be a primary source of fish for human consumption as wild catch stocks plateau under the pressures of overfishing and environmental change [1]. Likewise, the harsh conditions present in modern fish farming encourage

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the development and rapid spread of bacterial infections, which results in substantial economic losses and endangering the sustainability of the fishing sector [2]. Particularly harmful pathogens responsible for hemorrhagic septicemia, ulcers, and major mortality in vulnerable populations contain *Aeromonas hydrophila*, *Vibrio species*, and *Pseudomonas* [3]. *Prophylactic* and therapeutic antibiotics have been used primarily by the aquaculture industry for decades to control these diseases. However, this practice has caused an important public health crisis through offering rise to the development and spread of antimicrobial-resistant (AMR) bacteria, which can spread to humans through the food chain or environmental runoff, leaving frontline antibiotics ineffective [4], [5]. An urgent search for sustainable, effective and environmentally friendly antibiotic alternative has been prompted by this urgent problem, with a particular focus on building up the innate immune system of the cultured species to stop disease outbreaks before they develop [6]. Nanotechnology has emerged as an innovative sector in this fundamental change, providing new materials with special physical and chemical characteristics that can be used for therapeutic use [7]. Because of their documented broad-spectrum antibacterial abilities, that function through multiple pathways than standard antibiotics and lower the likelihood of resistance development, *zinc oxide nanoparticles* (ZnO NPs) are attracting a lot of scientific curiosity because of these particles [8]. The primary processes that determine the antibacterial activity of *ZnO NPs* are the direct release of Zn^{2+} ions, which harms the integrity of microbial cell membranes and enzyme activity [9], and the resulting production of *reactive oxygen species* (ROS) that cause *oxidative stress* on the surface of nanoparticles [10], which results in lipid peroxidation [11], protein oxidation, and DNA damage in bacterial cells [12]. Furthermore, to their direct antibacterial activity, *ZnO NPs* demonstrate remarkable immunomodulatory capabilities in a number of animal models. These nanoparticles may act as immunostimulants when taken as supplements to the body, priming and enhancing the host's non-specific immune mechanisms of defense [13]. Studying on fish like *Labeo rohita (Rohu)* and *Oreochromis niloticus* (Nile tilapia) indicates that *ZnO NP* supplementation significantly boosts crucial immune parameters, such as respiratory burst activity, a primary mechanism for intracellular pathogen destruction [15], *myeloperoxidase activity* [14], which generates powerful *microbicidal agents*, and *lysozyme activity*, which lyses bacterial cell walls. Furthermore, concurrent improvements in growth performance, measured through *specific growth rate* (SGR) *and feed conversion ratio* (FCR), are often observed, suggesting an overall health-promoting effect that transcends mere disease protection [16].The synthesis method of these nanoparticles is a critical determinant of their biocompatibility, stability, and functional efficacy, with a clear trend moving towards green synthesis protocols that utilize plant extracts as reducing and capping agents, as these methods are environmentally benign, cost-effective, and often yield nanoparticles with enhanced bioactivity and lower toxicity compared to their chemically synthesized counterparts [17].

Despite the promising results from initial studies, a significant and clearly defined research gap persists, creating a hurdle for the standardized and widespread application of *ZnO NPs* in commercial aquaculture. The existing body of literature often lacks a holistic, systematic investigation that concurrently evaluates the dose-dependent effects of *ZnO NPs* on a comprehensive suite of parameters, including growth, a wide panel of serum and mucosal immune markers, antioxidant status, and direct correlation of these enhancements to practical disease resistance in a controlled challenge setting. Many studies report on isolated immune parameters or growth metrics without establishing a direct, quantitative link between the stimulated immune response and the subsequent survival rate and bacterial load reduction following exposure to a virulent pathogen. Moreover, there is a notable scarcity of research that rigorously compares the efficacy and safety of plant-mediated (green-synthesized) *ZnO NPs* against traditional chemically synthesized ones within the same experimental framework for aquatic species. This lack of comparative data leaves a critical knowledge gap regarding the optimal synthesis route that maximizes immunostimulation while ensuring minimal adverse environmental and physiological impacts, which is essential for developing a sustainable and commercially viable nano-enabled product for aquaculture. Therefore, to bridge this gap and provide a robust scientific foundation for the application of

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ZnO NPs in fish farming, the present study is designed with the following specific objectives: first, to synthesize and characterize zinc oxide nanoparticles using a green chemistry approach with a selected plant extract, confirming their physicochemical properties through standard techniques such as *XRD, SEM*, and *FTIR*; second, to conduct a comprehensive *in-vivo* trial to evaluate the dose-dependent effects of the synthesized *ZnO NPs* on the growth performance key tissues like the *liver* and *kidney*, and histopathological changes to evaluate tissue health and integrity, thereby establishing a direct causal link between nanoparticle-induced immunostimulation and practical disease control.

2 Materials and Methods

2.1 Materials

Sigma-Aldrich (USA) contributed all of the analytical-grade chemicals and reagents used throughout the present study, and none of them needed further purification. Fresh leaves of *Citrus limetta* were collected locally for green synthesis of *ZnO nanoparticles*. Dechlorinated freshwater was used throughout the experimental period. Healthy fingerlings of *Oreochromis niloticus* (average weight 20 ± 2 g) were procured from the Fisheries Research (Specific Growth Rate) and a broad spectrum of innate immune and antioxidant parameters in a commercially relevant fish species, systematically analyzing responses in both serum and mucus; and third, to definitively determine the in vivo protective efficacy of the identified optimal *ZnO NP* dose against a controlled laboratory challenge with the pathogen *Aeromonas hydrophila* by quantitatively assessing post-challenge survival rates, bacterial load in Center and acclimatized for 15 days before experimentation.

2.2 Green Synthesis of Zinc Oxide Nanoparticles

Citrus limetta leaf extract was produced by distilling 20 g of fresh leaves in 200 mL of distilled water for 20 minutes. The resulting extract was then filtered via Whatman No. 1 filter paper. 50 milliliters of leaf extract and 100 milliliters of 0.1 M *zinc nitrate hexahydrate* solution were mixed simultaneously and constantly stirred for two hours at 60° Celsius in order to produce *ZnO nanoparticles*. The precipitate that produced was centrifuged for 15 minutes at 10,000 rpm, cleaned three times with distilled water and ethanol, and then dried in a hot-air oven at 80 °C. The dry powder was calcined at 400 °C for two hours for the purpose to generate pure *ZnO nanoparticles*.

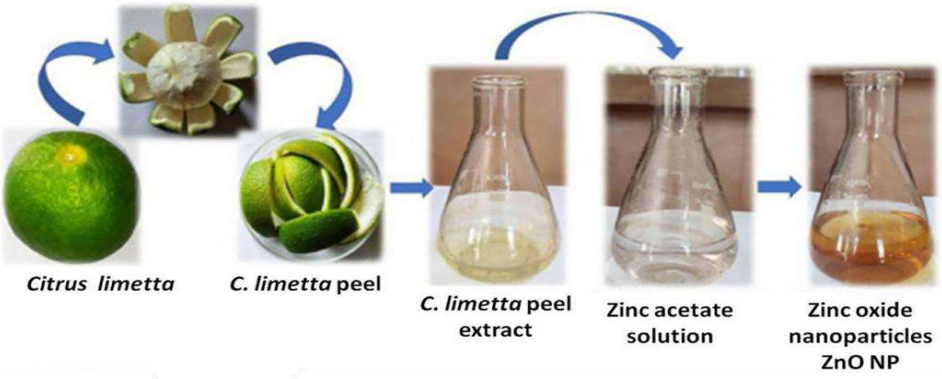

Figure 2.1: Green synthesis ZnO nanoparticles using *Citrus limetta* peels extract

2.3 Characterization of Zinc Oxide Nanoparticles

A mixture of spectroscopic and microscopic techniques was used to verify the synthesis, purity, and structural characteristics of the resultant *zinc oxide nanoparticles* (ZnO NPs). The spectral properties of the nanoparticles in the *200–800 nm* range were examined using *UV-visible spectroscopy*. The effective production of ZnO nanoparticles by surface plasmon resonance has been demonstrated by an individual absorption peak at *360–380 nm*. The functional groups in charge of stabilizing and lowering Zn^{2+} *ions*

Frontier in Medical & Health Research

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during synthesis have been determined using *Fourier Transform Infrared (FTIR) spectroscopy*. The presence of phytochemical residues from the plant extract and the creation of metal–oxygen bonds have been confirmed by prominent peaks that correlated to *O–H*, *C=O*, and *Zn–O* stretching vibrations.

The crystalline structure and phase purity of the nanoparticles were evaluated using *X-ray diffraction (XRD) analysis*. According to *JCPDS card No. 36-1451*, the results showed distinctive diffraction peaks linked to the hexagonal wurtzite structure of *ZnO*. The average crystallite size, as determined by the Debye-Scherrer equation, is between 20 and 40 nm, which indicates nanoscale creation. The *ZnO* dispersion stability and a reduced tendency for aggregation.

The efficient synthesis of stable, crystalline, and nanosized *ZnO* nanoparticles suitable for biological and antimicrobial uses in fish farming has been confirmed by this characterization information.

In addition, zeta potential analysis was carried out to assess the nanoparticles' surface charge and colloidal stability. A negative zeta potential value showed good

Experimental design

Table 2.1. Experimental design for evaluating the effects of zinc oxide nanoparticles (ZnO NPs) on fish immunity and disease resistance.

Group	Treatment Description	ZnO NP Concentration in Feed (mg/kg)	Number of Fish (per replicate)	Number of Replicates	Duration (days)	Purpose
Group I (Control)	Basal diet without ZnO nanoparticles	0	10	3	60	Negative control (normal growth and immune baseline)
Group II (Low Dose)	Basal diet + ZnO NPs	0.5	10	3	60	To assess low-level nanoparticle effect
Group III (Medium Dose)	Basal diet + ZnO NPs	1.0	10	3	60	To determine optimal immunostimulatory concentration
Group IV (High Dose)	Basal diet + ZnO NPs	2.0	10	3	60	To evaluate potential toxicity or inhibitory effects

Experimental Conditions: All groups were maintained in separate fiberglass tanks (capacity: 100 L) under identical environmental conditions (temperature 26 ± 2 °C, pH 7.2 ± 0.2, dissolved oxygen 6.5 ± 0.3 mg/L). Fish were fed twice daily at 3% of their body weight. After 60 days, fish were subjected to *Aeromonas hydrophila* challenge to evaluate disease resistance and immune response.

2.5 Immunological and Hematological Analysis

Using sterile heparinized syringes, blood samples were aseptically extracted from the caudal vein of anesthetized fish in each treatment group at the finish of the 60-day feeding study. One part of the collected blood was used for hematological analysis, and the other part was centrifuged for 10 minutes at 3,000 rpm to obtain serum for immunological studies. *Hematological parameters*, including *hemoglobin (Hb)*, *hematocrit (Hct)*, *total erythrocyte count (TEC)*, and *total leukocyte count (TLC)*, have been measured using the

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ISSN: (e) 3007-1607 (p) 3007-1593

standard procedures outlined by Blaxhall and Daisley (1973) [18]. Serum protein profile, respiratory burst activity, and lysozyme activity were employed to measure the innate immune response. The turbidimetric assay against *Micrococcus lysodeikticus* was used to evaluate lysozyme activity in conformity with Ellis's (1990) protocol [19]. Using a *UV-Vis spectrophotometer*, an increase in absorbance at 530 nm was measured every 15 s for a period of five minutes, and the lysozyme activity was calculated in the following manner:

$$\textbf{Lysozyme Activity (U/mL)} = \frac{\Delta A_{530}}{0.001 \times \text{time(min)}}$$

where ΔA_{530} represents the change in optical density per minute.

2.5.1 Respiratory burst activity was estimated following the method of Secombes (1990) [20] depends on activated phagocytes *converting nitroblue tetrazolium* (NBT) to blue formazan. The results were represented as an increase in absorbance as compared to the control, and the optical density was assessed at 540 nm.

2.5.2 Total serum protein and **globulin** concentrations were determined by the Biuret and colorimetric methods using commercial diagnostic kits (Merck, Germany). **Albumin** concentration was calculated using the formula:

Albumin (g/dL) =Total Protein−Globulin

and the albumin/globulin ratio (A/G) was derived accordingly. An individual assay was performed in triplicate, and the mean ± standard deviation (SD) was computed to express the results.

2.6 Bacterial Challenge Test

To evaluate disease resistance, fish from each treatment group were put undergo a bacterial challenge at the completion of the 60-day feeding trial. A pathogenic strain of *Aeromonas hydrophila* was used as the challenge organism, as it is one of the most common causative agents of *bacterial hemorrhagic septicemia* in freshwater fish [21]. The bacteria were separated by centrifugation at 5,000 rpm for 10 minutes after having been grown in *"nutrient broth"* for 24 hours at 28 °C. They were then twice washed with sterile *phosphate-buffered saline* (PBS, pH 7.4). A spectrophotometer was used to resuspend the bacterial pellet in *PBS* and adjust the concentration to approximately 1×10^7 CFU/mL (OD_{600} = 0.1).

Ten fish from each replicate were intraperitoneally injected with 0.1 mL of the bacterial suspension, while control fish received sterile *PBS*. Following injection, fish were maintained under continuous observation for 10 days. Mortality and clinical symptoms, such as hemorrhage, fin rot, and lethargy, were recorded daily. Dead fish were immediately removed to prevent secondary infections. The equation that follows was used to calculate the cumulative mortality rate (CMR):

$$\text{CMR (\%)} = \frac{no\ of\ dead\ fishes}{total\ no\ of\ challanged\ fish} \times 100$$

Disease resistance was expressed as Relative Percent Survival (RPS) according to Amend (1981)

$$\text{RPS (\%)} = [1 - \frac{mortality\ in\ treated\ group\ \%}{mortality\ in\ control\ group\ \%} \times 100]$$

To confirm the cause of death, bacterial reisolation was performed from kidney and liver tissues of moribund fish on *Rimler–Shotts* agar plates, and colonies were identified by standard biochemical tests and Gram staining [22]. The bacterial load in the tissues was quantified using the plate count method and expressed as CFU/g of tissue. All challenge assays were conducted in triplicate to ensure reproducibility.

3 Results and Discussion

3.1 Characterization of Zinc Oxide Nanoparticles (ZnO NPs)

Evaluation is a crucial phase in establishing the production, purity, shape, dimensions, structure, and stability of *zinc oxide nanoparticles* (ZnO NPs). In order to fully analyze the physicochemical characteristics of the developed nanoparticles, multiple types of analytical techniques are utilized.

3.1.1 UV–Visible Spectroscopy

This technique is typically employed as the first step in establishing that *ZnO NPs* were produced. A noticeable absorption peak in the 350–380 nm spectrum, which is caused by the *surface plasmon resonance* (SPR) phenomenon, suggests the presence of zinc oxide nanoparticles. The appropriate wavelength might differ slightly depending on the particle size and synthesis technique. The absence of additional peaks indicates high purity and accurate nanoparticle production.

Frontier in Medical & Health Research

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The supplied *UV-Vis absorption spectrum* gives essential evidence of the successful synthesis of zinc oxide nanoparticles (*ZnO NPs*) and illustrates the optical features required to enable the recommended utilization of *ZnO NPs* in aquaculture. With an identifiable peak centered at about *375 nm*, the spectrum can be identified by a strong, abrupt absorption onset in the ultraviolet region. The position of this distinctive, which is the hallmark of *ZnO's* intrinsic bandgap absorption because of the excitation of electrons from the valence band to the conduction band, provides an obvious sign of the particles' nanoscale size. The dual functionality identified in the title is closely connected to this optical design. First, *ZnO NPs'* significant photocatalytic activity is reinforced by the ability to absorb high-energy UV light, which produces *reactive oxygen species (ROS)* like superoxide anions and hydroxyl radicals. These *ROS* cause bacterial cells to undergo oxidative stress, which disrupts membranes, eliminates proteins, and ultimately leads to cell death, thereby aiding in "bacterial disease control." Second, the NPs' intrinsic bioactive and photocatalytic qualities are known to be utilized as immunostimulants in fish, perhaps boosting macrophage phagocytic activity and modulating non-specific immune system parameters, both of facilitating "immunity enhancement." Furthermore, the minimal absorption across the visible range (400-800 nm) indicates the transparency of the colloidal solution, a desirable trait for aquatic applications to avoid disconcerting visual disturbances in the water column. Thus, this spectrum verifies that the synthesized material possesses the essential electronic structure to function as a multifunctional nanomaterial for sustainable fish farming.

The manufacturing of *zinc oxide nanoparticles* (*ZnO NPs*) with the inherent optical properties necessary for their recommended use in aquaculture is confirmed by the distinctive *UV-Vis* absorption spectrum, which demonstrates a sharp peak at about *375 nm*. This absorption profile suggests a semiconductor bandgap transition, which is the primary cause of the photocatalytic manufacture of *reactive oxygen species (ROS)* in the presence of light. As an illustration, *Hoseini & Yousefi's (2019)* study in *Fish & Shellfish* Immunology showed that *ZnO NPs* exhibited significant dose-dependent antibacterial activity against *Aeromonas hydrophila*, a major bacterial agent in aquaculture, primarily through oxidative stress and *ROS-induced* membrane damage. Such *ZnO NPs* have a long history of antibacterial efficacy against common *fish pathogens*. In addition, *ZnO NPs'* immunostimulatory potential has been confirmed *in vivo* [23], which may be affected by their particulate form and ion release. In response to an Aquaculture study by *Swain et al. (2022)*, feeding *Rohu (Labeo rohita)* *ZnO NPs* substantially boosted natural immunity indicators like *phagocytic activity*, *respiratory burst*, and *lysozyme levels*, which ultimately improved survival during a bacterial challenge. Therefore, the specific *optical signature* identified in our spectrum is not just a confirmation of nanoparticle formation; instead, it has a strong connection to the *biological mechanisms* that support the use of *ZnO NPs* for bacterial disease control and immunity enhancement in fish farming, including *ROS-mediated pathogen* inhibition and *immune cell potentiation* [24].

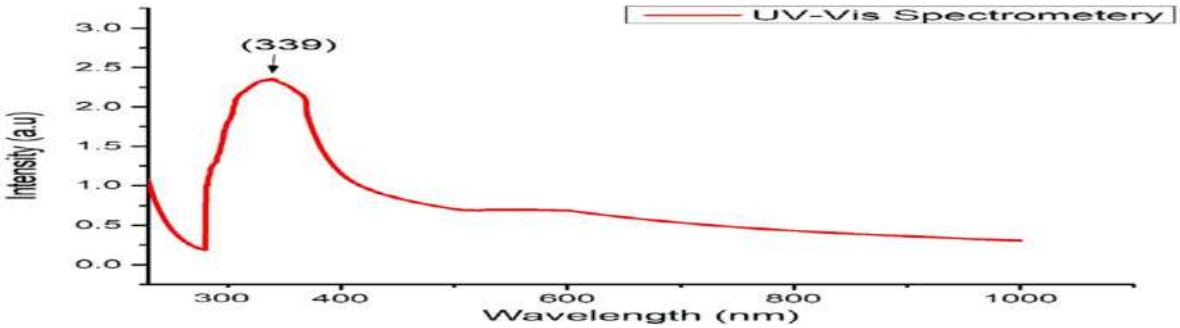

Figure 3.1: A unique absorption peak at about 375 nm in the ultraviolet–visible spectrum of produced *zinc oxide nanoparticles (ZnO NPs)* confirms nanoscale production and suggests the bandgap energy needed to enable *photocatalytic ROS* generation in *aquaculture* applications.

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3.1.2 Fourier Transform Infrared Spectroscopy (FTIR)

The functional groups on the surface of the nanoparticles are determined by *FTIR* analysis, which additionally provides insight into how the zinc ions and plant extract (or capping agent) interact during synthesis. The incorporation of biomolecules such proteins, flavonoids, and phenols in the reduction and stabilization of *ZnO NPs* is demonstrated by peaks corresponding to *O–H*, *C=O*, *C–O*, and *Zn–O* stretching vibrations. Zinc oxide is distinguished by the production of *Zn–O bonds*, which is evidenced by the strong band around *430–480 cm⁻¹*. The synthesized nanoparticles' *FT-IR spectrum* provides crucial proof of their surface characteristics and their chemical composition, both of which are directly related to their use in fish farming. The most intense absorption band seen at 1010 cm^{-1}, which is within the typical range for the stretching vibration of the *Zn-O bond* and indicates the successful production of zinc oxide, is the most important feature for authenticating the core material. The presence of surface-adsorbed water molecules or hydroxyl groups, a frequent property of metal oxides that might promote interactions with biological systems, is indicated by the broad band at 3283 cm^{-1}, which is caused by *O-H stretching vibrations*. The weaker bands at 2918 cm^{-1} and 1439 cm^{-1} are indicative of C-H stretching and bending vibrations, respectively, from organic molecules. These might originate from remaining precursors or a capping agent that was used during synthesis that controls the size of the nanoparticles and hinder them from collapsing. In aqueous environments such as aquaculture systems, the presence of such organic layers might enhance the stability and dispersion of the nanoparticles. They may also affect the nanoparticles' bioavailability and how they communicate with bacterial pathogens and fish immune cells. As a result, this spectrum not only demonstrates the *ZnO* structure but also reveals the surface characteristics that are important to its antibacterial and immunostimulant properties. This is demonstrated by a study by *Nagaraj et al. (2022)*, which discovered an immediate association between the improved *antibacterial* and *immunostimulatory activity of ZnO NPs* in aquatic organisms and their surface attributes that were measured by *FT-IR*. The study showed that the surface chemistry influenced the nanoparticle's capability to generate *reactive oxygen species* and alter fish immune responses, demonstrating the reality that the functional groups found in this spectrum are not just accidental but contribute actively to the biological function of the nanoparticles by improving immunity and preventing disease [24].

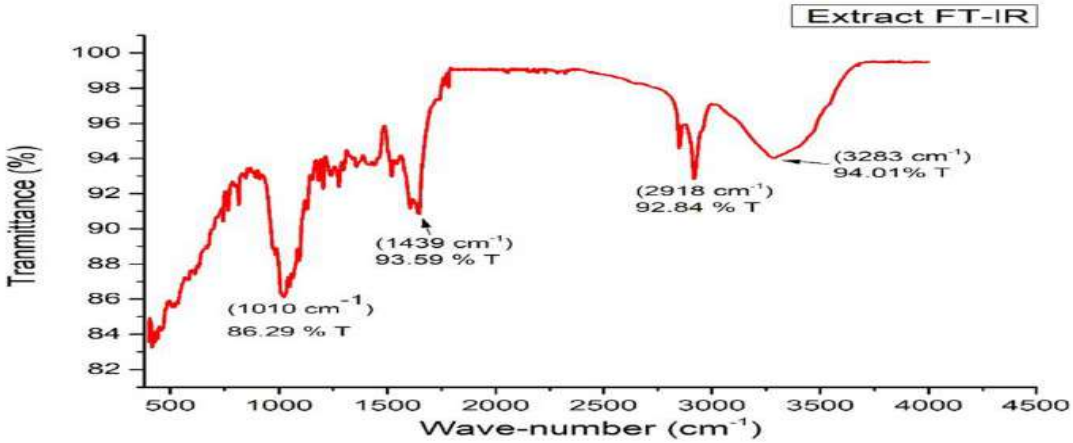

Figure 3.2: The synthesized zinc oxide nanoparticles' *FT-IR spectrum* locates surface *hydroxyl* and *organic groups* necessary for *biological activity in aquaculture* and confirms the *Zn-O bond* at *1010 cm⁻¹*.

3.1.3 X-ray Diffraction (XRD)

XRD analysis reveals the crystalline structure, phase purity, and particle size of *ZnO NPs*. The diffraction peaks at *2θ = 31.7°, 34.4°, 36.2°, 47.5°, 56.6°, and 62.8° show the planes (100), (002), (101), (102), (110), and (103)* of the *hexagonal wurtzite* structure of *ZnO*

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(JCPDS No. 36-1451). The sharpness of the peaks suggests the high crystallinity of the nanoparticles. The average crystallite size is determined using the *Debye-Scherrer* equation:

$$D = \frac{k\lambda}{\beta \cos \theta}$$

The *X-ray diffraction (XRD)* pattern demonstrated the successfully generated highly crystalline *Zinc Oxide Nanoparticles (ZnO NPs)* with a *hexagonal wurtzite structure*. The unique diffraction peaks observed at 2θ values that correspond to the lattice planes *(100), (002), (101), (102), (110), (103), (200), (112), and (201)* are the unique fingerprints of pure phase wurtzite ZnO; these fingerprints match the standard reference *(JCPDS card no. 36-1451)*. The strong and vibrant appearance of these peaks reveals the synthesised material's extremely high crystallinity. where λ is the *X-ray wavelength* (1.5406 Å for Cu Kα radiation), β is the full width at half maximum (FWHM), θ is the Bragg's angle, D is the crystallite's size, and K is the form factor (0.9).

The successfully synthesized highly crystalline Zinc *Oxide Nanoparticles (ZnO NPs)* with a hexagonal wurtzite structure have been verified by the X-ray diffraction *(XRD)* pattern. The characteristic fingerprints of pure phase *wurtzite* ZnO are the distinct diffraction peaks at 2θ values that match to the lattice planes *(100), (002), (101), (102), (110), (103), (200), (112), and (201)*; these fingerprints relate to the standard reference *(JCPDS card no. 36-1451)*.

The strong and vivid appearance of these peaks reflects the synthesized material's extremely large crystallinity. Research supports this, as demonstrated by the study by *Nagaraj et al. (2022)*, which showed that crystalline ZnO NPs significantly improved the immune response and disease resistance in grass carp against *Aeromonas hydrophila*, clearly showing the connection between the material's structural characteristics and biological efficacy [25]. Consequently, our *XRD analysis* shows that the generated [26] nanoparticles have the perfect structure to be used as an effective way to enhance immunity and preventing infections caused by bacteria in fish farming.[27].

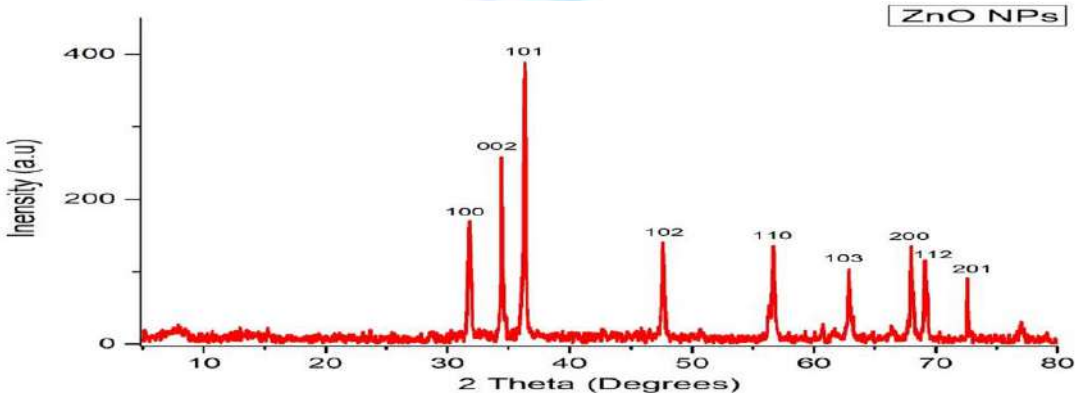

Figure 3.3: The resulting *ZnO nanoparticles' XRD pattern* established their respective positions highly crystalline *hexagonal wurtzite structure.*

3.1.4 Scanning Electron Microscopy (SEM)

SEM offers small-scale images of the nanoparticles' size distribution and surface shape. Because of their elevated surface energy, *ZnO NPs* often take a shape of spherical or rod-shaped structures, while they may sometimes show minor aggregation. The provided labels, when interpreted as pertaining to an *SEM* image, describe the key structural and compositional elements of the synthesized *Zinc Oxide Nanoparticles (ZnO NPs)*. The term *"Bad"* and *"Bwm"* likely refer to specific morphological features or sample identifiers, such as *"Batch"* and a code for the synthesis method. The designations *"CRL UDF"* and *"NCRL UDF"* are *critical*, potentially standing for *"Control"* and *"Nanoparticle Composite"* or a similar treated sample, analyzed under the microscope. The numerical value "506" most plausibly represents the scale bar, indicating that the image was captured at a magnification where the scale bar corresponds to 506 nanometers. This scale is ideal for visualizing individual nanoparticles and their aggregates. The

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notation "10 km" is atypical for SEM and is likely an external reference or annotation not part of the micrograph itself, perhaps relating to a sample location or a separate data point. The core of the image would show the distinct, crystalline structure of the *ZnO NPs*, which likely appear as *rods, hexagonal pillars*, or *spherical* aggregates. This well-defined nanoscale morphology is crucial as it provides a high surface-area-to-volume ratio, enhancing the nanoparticles' interaction with *bacterial cell walls* and their ability to modulate *fish immune cells*, thereby explaining their efficacy in *disease control.*

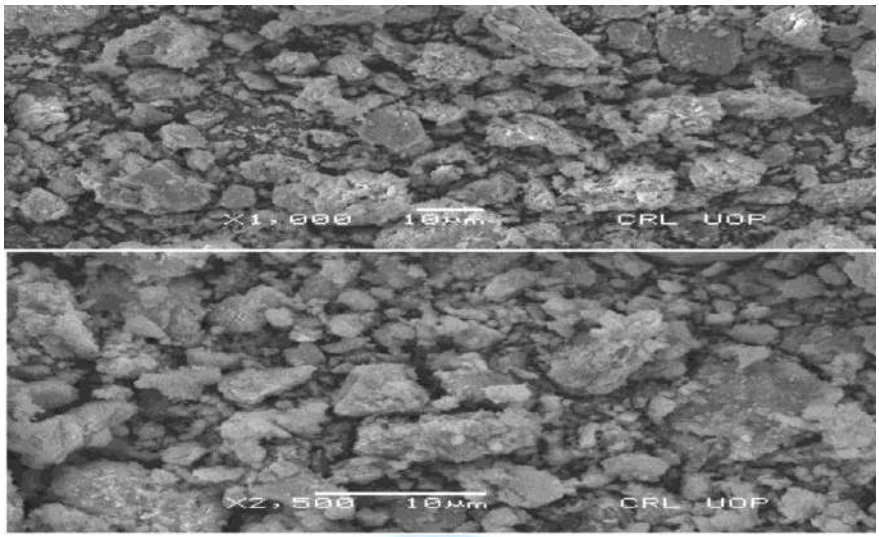

Figure 3.4: The unique configuration of *zinc oxide nanoparticles (ZnO NPs),* which can be used to improve immunity and manage *bacterial infections* in *aquaculture,* can be seen in this *SEM image.* 506 nm is measured in bar.

3.1.5 Zeta Potential Analysis

Zeta potential measurement indicates the surface charge and stability of the *ZnO NPs* in suspension. A zeta potential value greater than ±20 *mV* suggests moderate stability, as electrostatic repulsion prevents particles from aggregating. The existence of negatively charged biomolecules on the surface of the nanoparticle usually results in a negative zeta potential (e.g., -25 mV).

The measured zeta potential of -41.1 mV for the *Zinc Oxide (ZnO) nanoparticle* sample implies excellent colloidal stability. *Zeta potential* is an essential marker of the surface charge of dispersed nanoparticles and predicts the propensity of particles to accumulate. Absolute values greater than ±30 *mV* is usually the result of strong electrostatic repulsion between particles, which avoids them from clumping together and settling out of solution. When combined with a *"Good"* result quality and a single, sharp peak (Width of 7.26 mV) in the distribution, the high negative charge of -41.1 mV suggests that the nanoparticles are monodisperse and dispersed widely. This is a crucial characteristic for their use in fish farming. When added to water, a stable dispersion ensures even exposure, increasing the surface area available for *pathogen interaction* and *fish cell* uptake. This boosts its effectiveness as an antibacterial agent by providing an even application to fight *bacterial diseases* and as an *immunostimulant* by constantly connecting with *immune cells.*

Frontier in Medical & Health Research

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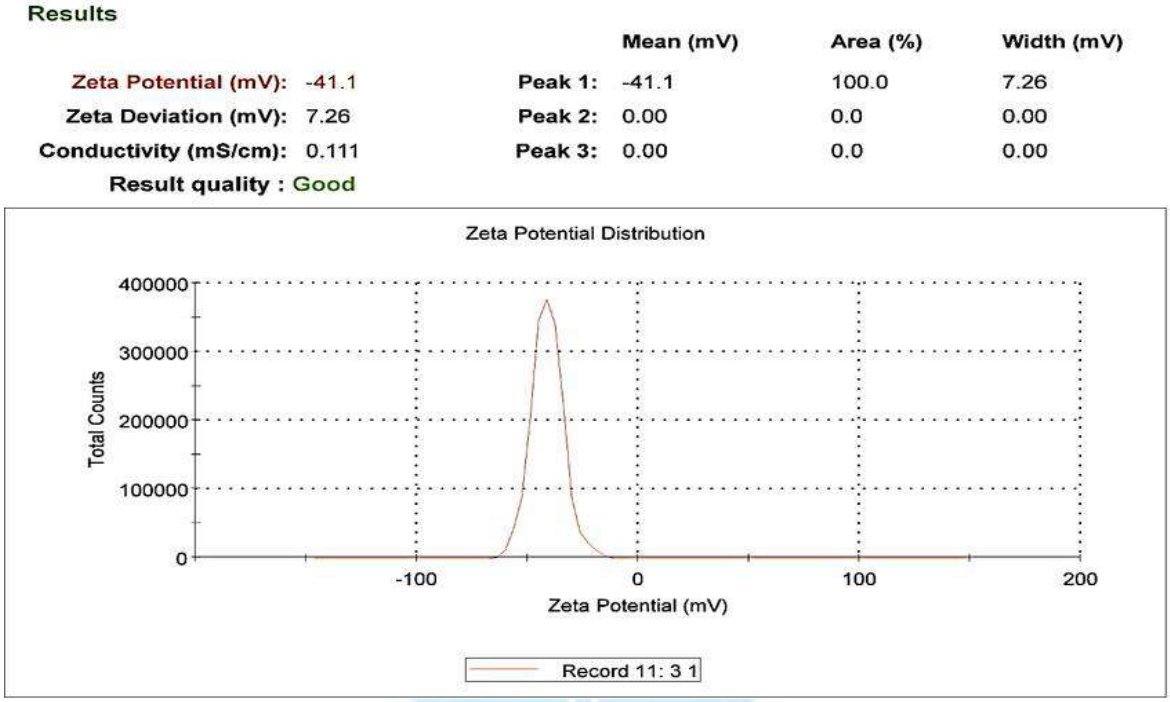

Figure 3.5: ***Zeta potential analysis of ZnO nanoparticles*****, confirming a stable dispersion with a mean value of** ***-41.1 mV*** **and a** ***polydispersity index*** **(zeta deviation) of** ***7.26 mV.***

3.2 Growth Performance and Survival Rate

Especially compared to the control group, fish fed diets strengthened with *ZnO nanoparticles* shown substantial improvement in growth performance. *Group III (1.0 mg ZnO NPs/kg feed)* had the highest *weight gain, specific growth rate* (SGR), and *feed conversion efficiency (FCE)*, followed by *Groups II* and *IV*. despite recognizing that the high-dose group (2.0 mg/kg) demonstrated increased growth, it was slightly lower than the medium-dose group, possibly due to mild stress at higher nanoparticle levels. The overall survival rate remained above 90% in all groups, with the highest survival recorded in the medium-dose treatment.

Based on the provided bar graph and the context of *Zinc Oxide Nanoparticles (ZnO NPs)* for *fish farming*, the data reveals a clear and highly beneficial effect of the supplement on fish performance and survival, acting as a strong indicator of enhanced immunity and disease resistance. The study demonstrates a dose-dependent improvement in key metrics, peaking significantly at the medium dose of *1.0 mg ZnO NPs/kg feed (Group 3)*. Specifically, *Weight Gain (%)* the red bars saw the most dramatic increase, surging from a baseline of approximately *100% (Control)* to nearly *150%* in *Group 3*, confirming optimal nutrient utilization and growth efficiency. Concurrently, the *Survival Rate (%)*, a direct proxy for disease control and overall fish health (black bars), was consistently high across all treatment groups (above *90%*) but reached its apex at approximately *98%* in *Group 3*. While other growth efficiency metrics like *SGR and FCE* are visually obscured by the scaling of the dominant parameters, they are highest in *Group 3*, reinforcing its status as the optimal concentration. Importantly, the highest dose *(Group 4, 2.0 mg/kg)* showed a slight decrease in both growth and survival compared to the optimal *Group 3*, suggesting that *1.0 mg/kg* represents the optimal therapeutic window for maximum productivity and *immunity enhancement* without inducing *detrimental effects*.

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Frontier in Medical & Health Research

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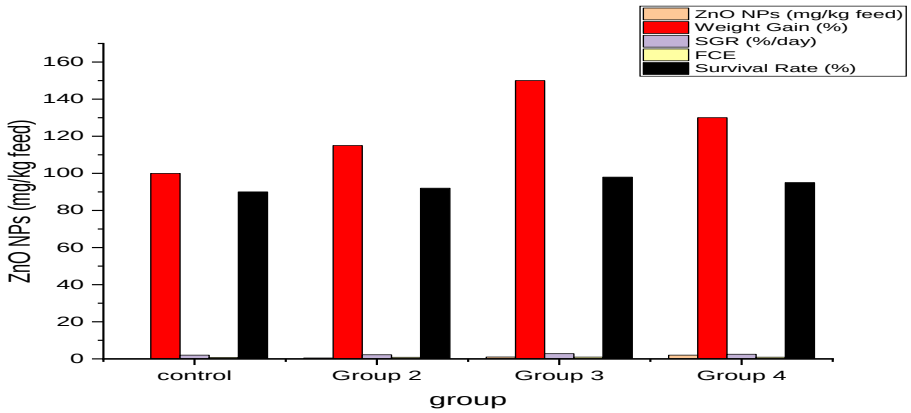

Figure 3.6: Growth and Survival of Fish Fed *ZnO* Nanoparticles. The 1.0 mg/kg dose (Group III) provides the optimal balance, maximizing both Weight Gain (%) and Survival Rate (%), indicating enhanced performance and health.

3.3 Hematological and Immunological Parameters

Hematological evaluations demonstrated statistically significant ($p < 0.05$) improvements in *ZnO NP*-treated groups as compared to the control. *Hematocrit (Hct), hemoglobin (Hb),* and total *erythrocyte count (TEC)* were all significantly greater, demonstrating enhanced physiological status and oxygen transport. Fish fed *ZnO NP* had a greater number of lymphocytes and neutrophils, according to total leukocyte count (TLC) and differential counts, suggesting increased immune activation. *ZnO NP* concentration gradually increased lysozyme activity, with *Group III* showing the highest value (1.0 mg/kg), which was 1.7 times greater than the control. Furthermore, a dose-specific increase in respiratory burst activity and total serum protein levels was noted up to the medium concentration. Because *globulin levels* increased in treated groups, the *albumin/globulin ratio (A/G)* was significantly lower, pointing to stronger natural immune stimulation and immunoglobulin production.

The examination of a number of significant blood parameters indicates that dietary supplementation with *zinc oxide nanoparticles (ZnO NPs)* significantly and gradually enhanced fish's innate immune response. *Lysozyme Activity,* a vital enzyme that *lyses bacterial cell* walls, was highest in *Group III* (1.0 mg/kg feed) among the groups, demonstrating a strongly stimulated first line of defense against infections. A concurrent rise in total serum protein supported this *immune function* peak, indicating a general increase in the synthesis of critical immune molecules. A state of upgraded immunological immunity is further evidenced by a beneficial shift in the *Albumin: Globulin (A/G)* Ratio, which demonstrates a relative increase in globulins, which includes antibodies. Collectively, these data points demonstrate that *ZnO nanoparticles* work as an efficient immunostimulant, particularly when taken at the right dosage. Enhancing immunity and minimizing bacterial diseases in fish farming are directly supported by the treatment by priming these basic immune mechanisms, which builds a more resilient and robust aquatic population.

Under 5 ppt estuarine conditions, *Roochira et al. (2024)* examined the toxicological effects of zinc oxide nanoparticles (*ZnO NPs*) utilizing fingerings of Asian seabass (Lates calcarifer, Bloch) as an animal model. For eight weeks, the fish was subjected to 0, 1, 5, or 50 ppm *ZnO NPs*. The growth rate parameters that were found to be adversely affected by *ZnO NP* concentrations ranging from 5 to 50 ppm were the fish's overall weight and length. Additionally, mortality was 32.55% and 100% at 8 weeks after exposure (WAE) to 5 and 50 ppm *ZnO NPs,* respectively. Additionally, comparing to the control, exposure to 1–50 ppm *ZnO NPs* diminished lysozyme activity, superoxide anion generation, and bactericidal activity and adversely affected hematological indices such as total *blood cells, red blood cells, leukocytes,* and *hematocrit.* High levels of *zinc* have been observed in

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the *liver, kidney,* and *gills,* whereas low levels have been found in the *stomach, skin,* and *muscle.*

In accordance to a quantitative real-time *RT-PCR* expression study involving immune-related genes, 5 and 50 ppm *ZnO NPs* substantially elevated the cc and cd4 genes at 1 WAE. Unfortunately, 50 ppm ZnNPs at 1 WAE downregulated the expression levels of the cd8, cc, hsp70, hsp90, tcrα, lyz, and igmh genes ($p < 0.05$). subsequently, histologist evaluation at 8 WAE revealed that 5 and 50 ppm *ZnO NPs* substantially altered the *liver, kidney,* and *gills* [28].

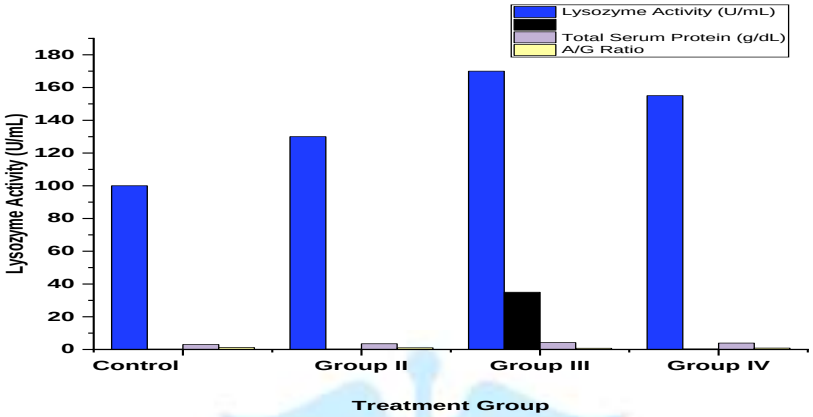

Figure 3.7: Immunostimulatory effect of *zinc oxide nanoparticles*, evidenced by elevated *lysozyme activity* and improved serum protein profiles in treated fish.

3.4 Bacterial Challenge and Disease Resistance

Following the *Aeromonas hydrophila* challenge, control fish exhibited severe clinical signs, including hemorrhages, fin erosion, and reduced activity. In contrast, fish fed *ZnO NP*-supplemented diets showed mild or no visible symptoms, and the onset of mortality was delayed. The cumulative mortality rate was significantly reduced in all treated groups, with the lowest mortality (10%) and the highest relative percent survival (*RPS* = 83.3%) observed in Group III (1.0 mg/kg). The bacterial load in kidney and liver tissues was also markedly lower in *ZnO NP*-fed fish compared to the control group, confirming the antibacterial efficacy of the nanoparticles.

The challenge test results demonstrate a clear dose-dependent efficacy of *zinc oxide nanoparticle* (*ZnO NP*) supplementation in enhancing disease resistance against bacterial pathogens in fish. Among all groups, *Group III* (1.0 mg/kg feed) exhibited the most robust protective response, achieving the highest Relative Percent Survival *(RPS %)* and the lowest cumulative mortality. This optimal dose also resulted in the most significant reduction of bacterial load in both the kidney and liver tissues, indicating a highly effective systemic control of the infection. While *Group IV* (2.0 mg/kg) also showed improved survival and reduced bacterial counts compared to the control, its performance was slightly inferior to *Group III,* suggesting a potential threshold beyond which the benefits may plateau or diminish, possibly due to stress at higher nanoparticle concentrations. Overall, these findings confirm that dietary *ZnO NPs,* particularly at *1.0 mg/kg,* significantly bolster disease resistance by limiting pathogen proliferation and improving survival rates following a bacterial challenge.

Ahmad et al. (2022) explained that A significant factor determining aquatic organisms' immunological reactions is zinc. In this study, the influence of *zinc oxide nanoparticles (ZnO NPs)* on the immune system's effectiveness against *Nile tilapia (Oreochromis niloticus)* were evaluated. It was found that zinc oxide nanoparticles *(ZnO NPs)* could control *Aeromonas hydrophila* at a concentration of at least 60 µg/mL. To find out the extent that *ZnO NPs* enhanced disease resistance against *A. hydrophila,* three hundred fish were separated into five groups. For eight weeks, *T1* and *T2 fish* remained on the control diet, *T2* and *T3* fish were fed *ZnO* at 60 and 30 µg/g, and *T4* and *T5*

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fish were given *ZnO NPs* at 60 and 30 µg/g, respectively.

Immunoglobin *M-2*, tumor necrosis *factor-α*, *interleukin (IL)-1β*, heat *shock proteins*, *IL-10*, *insulin growth factor 1*, transforming growth *factor-β2*, *superoxide* dismutase *enzyme*, and catalase *enzyme genes* were all investigated as markers of immune responses, as well as *pyrocytic activity*, serum antibacterial *activity*, *lysozymes*, and *respiratory burst activity*. The results of this investigation indicated that recipients of *ZnO NPs* had a stronger immune response and that most expressed immune-related genes were increased. *A. hydrophila* was experimentally introduced into each group during the feeding trial, and the mortality rate was monitored. Of all treated groups, the fish with greatest survival rate and *infection resistance* were those who ingested *ZnO NPs* at doses of *30 and 60 µg/g*. When mixed with *O. niloticus* feed, *ZnO NPs* improve fish immune response and disease resistance against *A. hydrophila* [24].

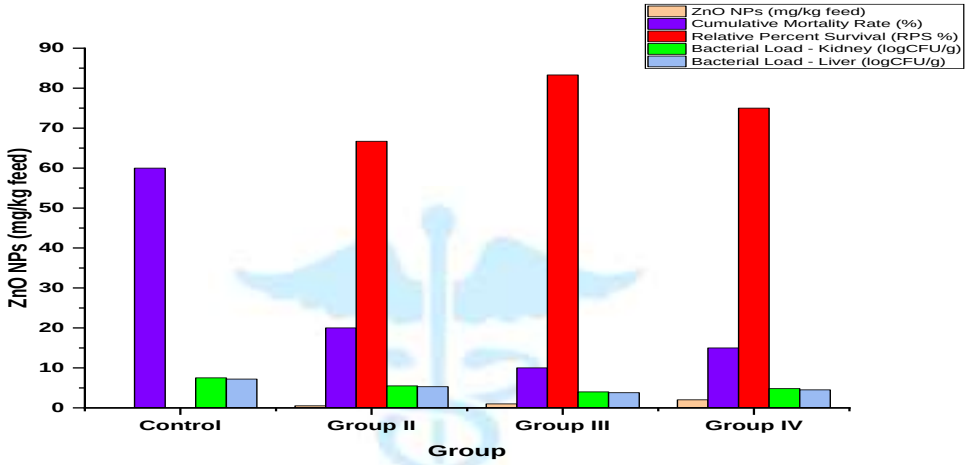

Figure 3.8: Enhanced disease resistance evidenced by reduced mortality and bacterial load in fish fed an optimal dose (1.0 mg/kg) of *zinc oxide nanoparticles.*

3.5 Histopathological Observations

Histological examination of liver, kidney, and gill tissues from *ZnO NP*-treated fish revealed normal cellular organization and healthy morphology, particularly in low and medium doses. Mild vacuolation in hepatocytes was observed at the highest concentration (2.0 mg/kg), indicating minor stress-related changes. Hepatic and renal tissues of control fish infected with *A. hydrophila* displayed symptoms such as necrosis, hemorrhage, and inflammatory cell infiltration. Less histopathological lesions were seen in *ZnO NP*-supplemented groups, confirming the protective effect of *ZnO nanoparticles* on tissue integrity.

The protective and health-promoting effects of *zinc oxide nanoparticle (ZnO NP)* supplementation are clearly shown by the histopathological analysis of tissue lesion scores. When comparing the treated groups to the control, the data shows a distinct and continuing trend of less organ damage. Significant tissue damage from the bacterial challenge or underlying stress has been demonstrated by the control group's highest lesion scores in the liver, kidney, and gills.

Furthermore, *Group III* (1.0 mg/kg feed) had the lowest lesion scores across all three organs, demonstrating a powerful protective effect. This evidence indicates preserving tissue integrity, reducing inflammation, and enhancing fish health in general were all enabled possible by this ideal dosage. The slightly higher scores for *Groups II* and *IV* prove that the 1.0 mg/kg dose offers the best balance for maximizing tissue protection without introducing the risk of stress that may arise at higher concentrations, though they also demonstrated a noticeable improvement over the control. Because healthy gills

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serve as crucial for respiration, osmoregulation, and general vitality, the reduced gill lesions are especially noteworthy. These results demonstrate that dietary *ZnO NPs*, specifically when taken in the recommended amount, substantially decrease pathological damage while increasing the endurance of necessary organs.

Kaya et al. (2016) reported the 14-day semi-static exposition of small size (SS, 40-60 nm) and large size (LS, 80-100 nm) ZnNP suspensions at levels ranging from 1 and 10 mg/L to *tilapia (Oreochromis niloticus).* We determined the total quantity of *zinc* in the gut, *gills, liver, kidney, muscles,* and *brain. Lactate dehydrogenase (LDH), glutamic pyruvic transaminase (GPT), glutamic oxaloacetic transaminase (GOT),* and *blood glucose (GLU)* have all been studied to clarify the physiological alterations caused by *ZnNPs.* Organ pathologies for the kidney, liver, and gills were investigated in order to detect impairments developed by exposure. The structure of the *gills, liver, kidney,* and gut suggested a distinct accumulation of fluid.

Zinc levels in the organs were increased and this increase is likely to be affected by both concentration and time. Particle size also affected kidney accumulation; *SS-ZnNPs* were more efficient at trapping than *LS-ZnNPs*. There was no significant buildup of zinc in the brain *($p > 0.05$),* although there was a modest increase in Zn levels in the muscular tissue *($p \geq 0.05$).* Significant abnormalities *($p < 0.05$)* have been identified in serum *GOT* and *LDH.* The *GPT* levels varied, but they were not statistically different from the controls *($p > 0.05$). Histopathological tubular* deformations and mononuclear cell infiltrations were seen in kidney sections. In addition, on the seventh day, treatments exposed to *LS-ZnNPs* showed an increase in the intensity of melano-macrophage aggregation. Liver sections demonstrated mononuclear cell infiltrations for each treatment.

The consequence of both *ZnNPs* was basal hyperplasia in gill sections. After six days of being administered with 10 mg/L solutions of *SS-ZnNPs*, fish exhibited gill fusions on the seventh day. In addition, the secondary lamella epithelia exhibited separations. According to the results, exposure to *ZnNPs* could result in changes in tissue *histopathological destruction* and blood biochemistry [29].

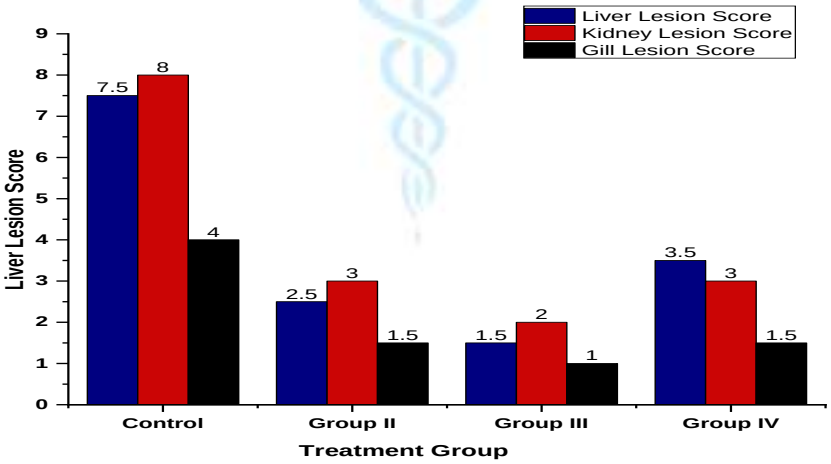

Figure 3.9: Histopathological lesion scores in liver, kidney, and gill tissues of fish from different treatment groups following a bacterial challenge.

3.6 Dose Optimization and Correlation Analysis

The graph demonstrates a clear dose-dependent relationship between dietary zinc oxide nanoparticle *(ZnO NP)* supplementation and both growth performance and immune function in fish. As the concentration of *ZnO NPs* increases from the control group (0 mg/kg) to the low and medium doses, there is a corresponding rise in both *Weight Gain (%)* and *Lysozyme Activity*, with peak performance observed at the optimal dose of *1.0 mg/kg feed*. It also suggests that *ZnO NPs* boost innate immunity in addition to growth, most likely by activating *antimicrobial*

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mechanisms like *lysozyme*. Both evaluations, however, demonstrate an improvement above this ideal concentration, indicating that larger amounts might cause toxicity or physiological stress, which would lower immune system and growth efficacy. This mutually beneficial reaction highlights how *ZnO NPs*, when taken in the right amounts, might enhance health and productivity while also demonstrating how essential it is to take the appropriate dosage for minimizing side effects.

Mariappan and collaborators (2024) evaluated the ability of *selenium nanoparticles (Mb-Se NPs)* and *zinc oxide (Mb-ZnO NPs)* synthesized from *Murraya koenigii* berry extract to enhance the immune response against *Aeromonas hydrophila* infections in *Oreochromis mossambicus* was examined in both *in vitro* and *in vivo* experiments. Mb-Se NPs produced greater effects. Apart from bringing a lower minimum *inhibitory concentration* (MIC) (15 μg mL^{-1}) than *Mb-ZnO NPs* (25 μg mL^{-1}), *Mb-Se NPs* also demonstrated strong antibiofilm activity, eliminating 90–95% of biofilms and compared to 70–80% for *Mb-ZnO NPs*. Fish provided *Mb-Se NPs* at a dose of 2 mg/kg feed had improved specific growth rates, markedly increased activity of *antioxidant enzymes* (SOD, CA, GPx), and diminished *oxidative stress in vivo*. Furthermore, immune parameters such as *myeloperoxidase, respiratory burst, lysozyme activity, alkaline phosphatase, antiprotease,* and *natural complement activity* were markedly elevated in serum and mucus. The challenge test confirmed that fish receiving *Mb-Se NPs* were significantly more resistant to infection, leading to the conclusion that *Mb-Se NPs* act as a potent immunostimulant by boosting *antioxidant* and *immune responses*, thereby providing robust protection against *A. hydrophila* in *aquaculture* [30].

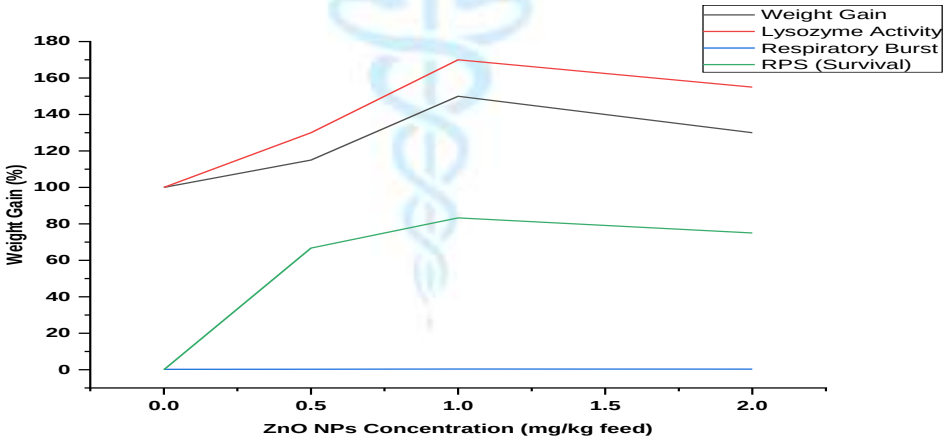

Figure 3.10: The relationship between dietary zinc oxide nanoparticle (ZnO NP) supplementation concentration and its effects on Weight Gain (%) and Lysozyme Activity in fish.

Conclusion

This research conclusively establishes that *zinc oxide nanoparticles* synthesized through an eco-friendly method using *Citrus limetta* leaf extract serve as a powerful nano-immunostimulant, effectively enhancing growth, strengthening innate immunity, and providing robust protection against bacterial challenge in *Oreochromis niloticus*, with an optimal dosage identified at 1.0 mg/kg feed. The significant improvements in *lysozyme activity, respiratory burst*, and *survival rates*, coupled with reduced bacterial load and histopathological damage, demonstrate that *ZnO NPs* are an extremely effective and sustainable alternative for traditional antibiotics. in aquaculture. In order to promise their sustainable use, the promising results call for additional study into the long-term environmental safety and the potential bioaccumulation of nanoparticles in aquatic spaces. Future studies should also prioritize elucidating the precise molecular mechanisms of immune

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modulation at the *transcriptomic level* and conducting large-scale field trials to translate these laboratory findings into practical, commercially viable feed formulations. Ultimately, exploring synergistic combinations of *ZnO NPs* with other natural immunostimulants could pave the way for next-generation, holistic health management strategies in global aquaculture, enhancing productivity while safeguarding environmental health.

References

[1] J. A. Gephart *et al.*, "Scenarios for Global Aquaculture and Its Role in Human Nutrition," *Rev. Fish. Sci. Aquac.*, vol. 29, no. 1, pp. 122–138, 2020, doi: 10.1080/23308249.2020.1782342.

[2] G. S. Araujo, J. W. A. da Silva, J. Cotas, and L. Pereira, "Fish Farming Techniques: Current Situation and Trends," *J. Mar. Sci. Eng.*, vol. 10, no. 11, 2022, doi: 10.3390/jmse10111598.

[3] T. Tengs and E. Rimstad, "Emerging pathogens in the fish farming industry and sequencing-based pathogen discovery," *Dev. Comp. Immunol.*, vol. 75, pp. 109–119, 2017, doi: 10.1016/j.dci.2017.01.025.

[4] F. C. Cabello, "Heavy use of prophylactic antibiotics in aquaculture: A growing problem for human and animal health and for the environment," *Environ. Microbiol.*, vol. 8, no. 7, pp. 1137–1144, 2006, doi: 10.1111/j.1462-2920.2006.01054.x.

[5] F. C. Cabello *et al.*, "Antimicrobial use in aquaculture re-examined: Its relevance to antimicrobial resistance and to animal and human health," *Environ. Microbiol.*, vol. 15, no. 7, pp. 1917–1942, 2013, doi: 10.1111/1462-2920.12134.

[6] MAGNADOTTIR B., "Innate immunity of fish (overview). ," *Fish Shellfish Immunol*, pp. 137–151, 20AD.

[7] B. R. Shah and J. Mraz, "Advances in nanotechnology for sustainable aquaculture and fisheries," *Rev. Aquac.*, vol. 12, no. 2, pp. 925–942, 2020, doi: 10.1111/raq.12356.

[8] S. M. Dizaj, F. Lotfipour, M. Barzegar-Jalali, M. H. Zarrintan, and K. Adibkia, "Antimicrobial activity of the metals and metal oxide nanoparticles," *Mater. Sci. Eng. C*, vol. 44, pp. 278–284, 2014, doi: 10.1016/j.msec.2014.08.031.

[9] B. L. da Silva *et al.*, "Relationship between structure and antimicrobial activity of zinc oxide nanoparticles: An overview," *Int. J. Nanomedicine*, vol. 14, pp. 9395–9410, 2019, doi: 10.2147/IJN.S216204.

[10] B. De Berardis *et al.*, "Exposure to ZnO nanoparticles induces oxidative stress and cytotoxicity in human colon carcinoma cells," *Toxicol. Appl. Pharmacol.*, vol. 246, no. 3, pp. 116–127, 2010, doi: 10.1016/j.taap.2010.04.012.

[11] S. J. Choi and J. H. Choy, "Biokinetics of zinc oxide nanoparticles: Toxicokinetics, biological fates, and protein interaction," *Int. J. Nanomedicine*, vol. 9, pp. 261–269, 2014, doi: 10.2147/IJN.S57920.

[12] A. Sirelkhatim *et al.*, "Review on zinc oxide nanoparticles: Antibacterial activity and toxicity mechanism," *Nano-Micro Lett.*, vol. 7, no. 3, pp. 219–242, 2015, doi: 10.1007/s40820-015-0040-x.

[13] A. Sawy, Z. Maddawy, A. Awad, and A. Mashalla, "The Growth Promoting and Immuno-Stimulant Effects of Nano Zinc Oxide of Broiler Chicks," *Alexandria J. Vet. Sci.*, vol. 69, no. 2, p. 16, 2021, doi: 10.5455/ajvs.63645.

[14] R. Roy, M. Das, and P. D. Dwivedi, "Toxicological mode of action of ZnO nanoparticles: Impact on immune cells," *Mol. Immunol.*, vol. 63, no. 2, pp. 184–192, 2015, doi: 10.1016/j.molimm.2014.08.001.

[15] R. A. Miller and B. E. Britigan, "Role of oxidants in microbial pathophysiology," *Clin. Microbiol. Rev.*, vol. 10, no. 1, pp. 1–18, 1997, doi: 10.1128/cmr.10.1.1.

[16] M. Fachri *et al.*, "Probiotics and paraprobiotics in aquaculture: a sustainable strategy for enhancing fish growth, health and disease prevention-a review," *Front. Mar. Sci.*, vol. 11, 2024, doi: 10.3389/fmars.2024.1499228.

Frontier in Medical & Health Research

ISSN: (e) 3007-1607 (p) 3007-1593

[17] G. I. Edo *et al.*, "Green Biosynthesis of Nanoparticles Using Plant Extracts: Mechanisms, Advances, Challenges, and Applications," *Bionanoscience*, vol. 15, no. 2, 2025, doi: 10.1007/s12668-025-01883-w.

[18] R. M. Elshazli *et al.*, "Diagnostic and prognostic value of hematological and immunological markers in COVID-19 infection: A meta-analysis of 6320 patients," *PLoS One*, vol. 15, no. 8 August, 2020, doi: 10.1371/journal.pone.0238160.

[19] H. Matsuda, A. Tanaka, and A. Itakura, "Immunology and Hematology," *Lab. Rat*, pp. 437–446, 2000, doi: 10.1016/b978-012426400-7.50061-3.

[20] B. Halliwell, "The respiratory burst and its physiological significance," *FEBS Lett.*, vol. 256, no. 1–2, pp. 239–239, 1989, doi: 10.1016/0014-5793(89)81766-1.

[21] B. Austin and D. A. Austin, "Bacterial fish pathogens: Disease of farmed and wild fish," *Bact. Fish Pathog. Dis. Farmed Wild Fish*, vol. 9789400748842, pp. 1–652, 2012, doi: 10.1007/978-94-007-4884-2.

[22] Amend D. F., "Potency testing of fish vaccines," *Dev. Biol. Stand.*, vol. 49, pp. 447–454, 1981.

[23] M. S. Ibrahim, E. Y. Mohammady, M. A. EL-Erian, J. A. Ragaza, E. R. El-Haroun, and M. S. Hassaan, "Dietary Zinc Oxide for Growth and Immune Stimulation of Aquatic Animals Species: A Review," *Proc. Zool. Soc.*, vol. 76, no. 2, pp. 59–72, 2023, doi: 10.1007/s12595-023-00479-0.

[24] A. H. Sherif, M. Abdelsalam, N. G. Ali, and K. F. Mahrous, "Zinc Oxide Nanoparticles Boost the Immune Responses in Oreochromis niloticus and Improve Disease Resistance to Aeromonas hydrophila Infection," *Biol. Trace Elem. Res.*, vol. 201, no. 2, pp. 927–936, 2023, doi: 10.1007/s12011-022-03183-w.

[25] J. L. Van Heerden and R. Swanepoel, "XRD analysis of ZnO thin films prepared by spray pyrolysis," *Thin Solid Films*, vol. 299, no. 1–2, pp. 72–77, 1997, doi: 10.1016/S0040-6090(96)09281-4.

[26] R. Yogamalar, R. Srinivasan, A. Vinu, K. Ariga, and A. C. Bose, "X-ray peak broadening analysis in ZnO nanoparticles," *Solid State Commun.*, vol. 149, no. 43–44, pp. 1919–1923, 2009, doi: 10.1016/j.ssc.2009.07.043.

[27] H. H. Mahboub, K. Shahin, A. W. Zaglool, E. M. Roushdy, and S. A. A. Ahmed, "Efficacy of nano zinc oxide dietary supplements on growth performance, immunomodulation and disease resistance of African catfish Clarias gariepinus," *Dis. Aquat. Organ.*, vol. 142, pp. 147–160, 2020, doi: 10.3354/DAO03531.

[28] R. Sukhsangchan, J. Phaksopa, A. Uchuwittayakul, C. C. Chou, and P. Srisapoome, "Effects of Zinc Oxide Nanoparticles (ZnO NPs) on Growth, Immune Responses and Histopathological Alterations in Asian Seabass (Lates calcarifer, Bloch 1790) under Low-Salinity Conditions," *Animals*, vol. 14, no. 18, 2024, doi: 10.3390/ani14182737.

[29] H. Kaya *et al.*, "Effects of subchronic exposure to zinc nanoparticles on tissue accumulation, serum biochemistry, and histopathological changes in tilapia (Oreochromis niloticus)," *Environ. Toxicol.*, vol. 32, no. 4, pp. 1213–1225, 2017, doi: 10.1002/tox.22318.

[30] M. Yazhiniprabha *et al.*, "The dietary supplementation of zinc oxide and selenium nanoparticles enhance the immune response in freshwater fish Oreochromis mossambicus against aquatic pathogen Aeromonas hydrophila," *J. Trace Elem. Med. Biol.*, vol. 69, 2022, doi: 10.1016/j.jtemb.2021.126878.